Analysis of diosgenin content and pharmacological exploration of critically endangered medicinal herb-*Trillium govanianum* (Nagchhatri)

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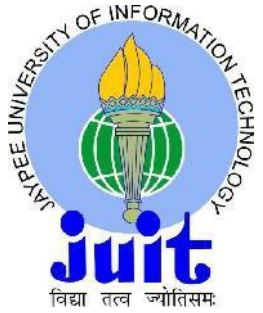

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Analysis of diosgenin content and pharmacological exploration of critically endangered medicinal herb- *Trillium govanianum* (Nagchhatri)

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Introduction

Trillium govanianum Wall. ex D. Don, popularly known as Nagchhatri (Trilliaceae), is an important medicinal herb found in North-West Himalayan region of India. It is a threatened plant species found at an altitude of 2400-3500 m above msl (Vidyarthi et al. 2013). The plant is a small herb which propagates through roots and seeds. The underground part of the plant, i.e. rhizome is key material of trade containing trillarin, which on hydrolysis yields diosgenin – a corticosteroid hormone. Reportedly, the cortico-steroid hormone isolated from the plant is used in various preparations like sex hormones; cortisone and allied preparation used in rheumatism, birth control, regulation of menstrual flow and in stomach related problems (Chauhan 1999) (Fig. 1).

Fig. 1 Mature field grown plants of *T. govanianum*

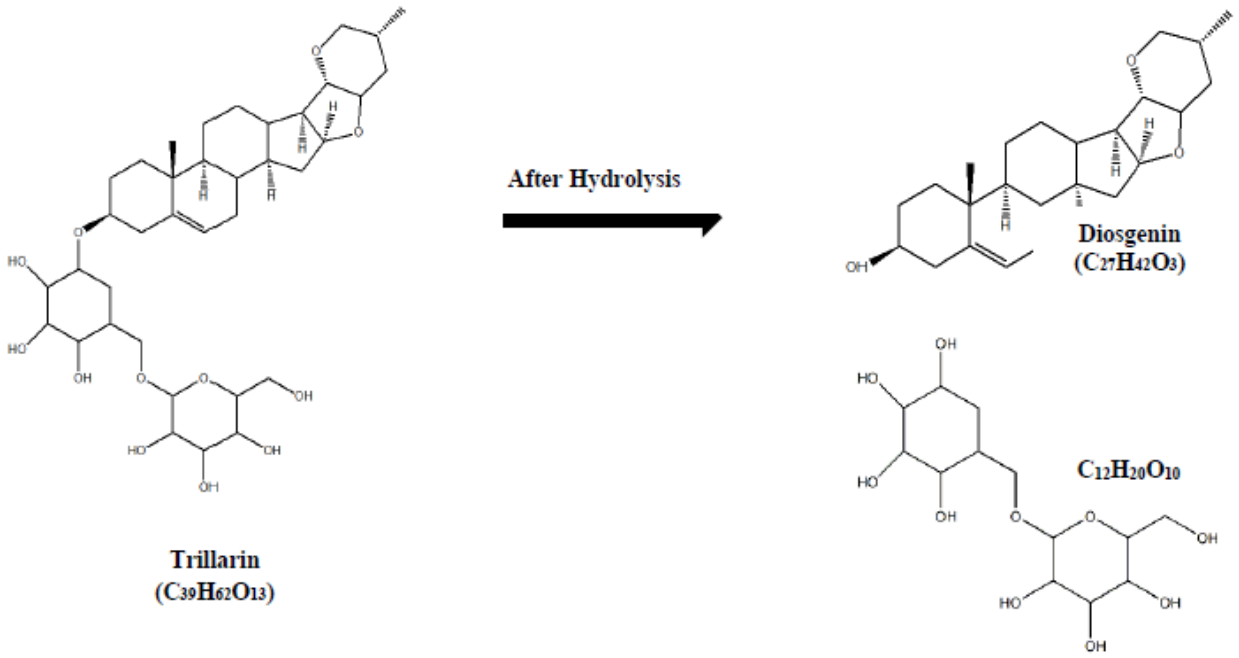


Fig. 2. A schematic hypothesized view for production of Diosgenin from Trillarin after hydrolysis

Relentless collection and smuggling *Trillium govanianum* because of its price (Rs 2,500- 5,000) will lead to extinction. At present, natural diosgenin is extracted from Trigonella species and the diosgenin content varies from 0.01% to 0.5% in aerial parts and seeds (Dangi, Misar et al. 2014). Diosgenin content varies in *Dioscorea* spp with *Dioscorea polygonoides* (0.2%); *Dioscorea althaeoides* (0.2-2.3%); *Dioscorea prazeri* (1.92%); *Dioscorea villosa* (1.3%); *Dioscorea zingiberensis* (0.18-0.55%) (Niño, Jiménez et al. 2007). Production of diosgenin from *Dioscorea* spp is both time consuming and costly because tubers require 3 years to grow to mature size (Šavikin-Fodulović, Grubišić et al. 1998, Acharya, Thomas et al. 2008, Zerbino and Birney 2008). Moreover, these raw materials are in short supply and production strategies of Trigonella and Dioscorea are not enough for meeting the present demand, so there is a need for alternative source for steroids. *T. govanianum* can serve as an alternative for higher production of diosgenin in shorter growing cycle (Vidyarthi, Samant et al. 2013). Due to immense medicinal importance, small population, limited niche, low degree of genetic diversity, low set rate of seed, reckless collection and escalating industrial demands, *T. govanianum* has been listed as an endangered medicinal plant species by the International Union for Conservation of Nature and Natural Resources (HU- Tian-yin and QIAN Li-hua 2009). Hence and immediate attention is necessary for conservation,

micropropagation, cultivation and *in vitro* production of secondary metabolites from *T. govanianum*.

T. govanianum is less explored so far the identification of govanoside, new steroidal saponins, and other components such as borassoside and pennogenin, which have been explored for antioxidant and anticancer activities (Shafiq ur, Ismail et al. 2015). These secondary metabolites produced in this species are analyzed specially for diosgenin, which is produced through steroid biosynthetic pathway. Recently, GC/MS analysis for rhizomes of *T. govanianum* has been carried out to investigate the phytochemical constituents & explored its antioxidants, anticancer activities (Ur-Rahman et al., 2015).

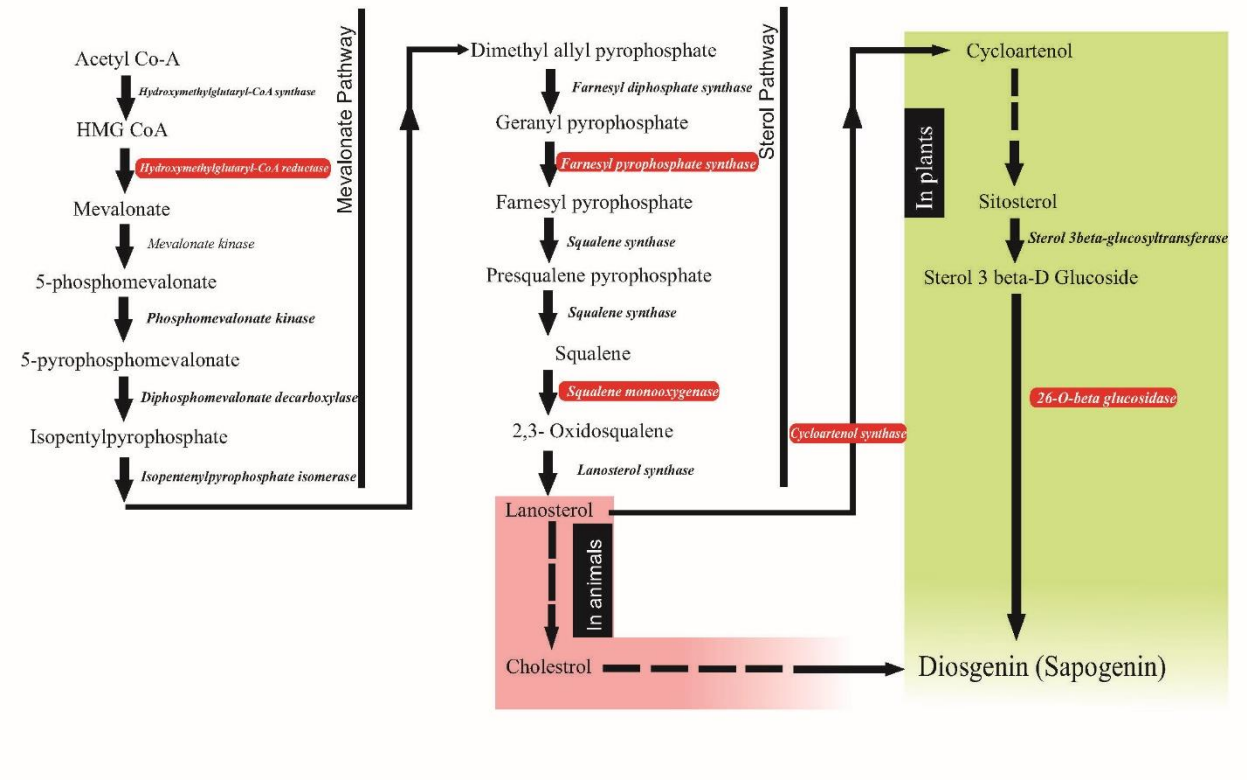


Fig. 3 Predicted pathway for diosgenin biosynthesis in *T. govanianum* (Vaidya et al. 2013; Diarra et al. 2013)

The availability of predicted pathway for diosgenin synthesis by Vaidya et al. (2013) and Diarra et al. (2013) helped us in taking the lead for molecular exploration in *Trillium govanianum* (Fig. 3). We have carried out RSM technology for extraction and improving the diosgenin content and also studied the biological activity of crude extracts for toxicity, anti-cancerous activity and anti-fertility studies on female rat model. Also analysis of *T. govanianum* from different altitudinal

ranges can provide information on proper selection of plant material for propagation, conservation and to be used for genetic intervention strategies. Therefore, the current work was undertaken with the following objectives:

Objective 1: Optimization of diosgenin extraction from *Trillium govanianum.*

Objective 2: Discerning the expression status of steroidal pathway genes for diosgenin biosynthesis in *T. govanianum.*

Objective 3: To investigate anti-cancerous & anti-fertility potential of *T. govanianum* extract.

Material and methods

Plant material

Twelve accessions of *T. govanianum* were collected from various areas from Sonmarg, Jammu & Kashmir and high altitudes of Chamba, Lahaul & Spiti areas of Himachal Pradesh in the month of August 2014.

Table 1: *T. govanianum* accessions from different geographical locations of H.P and J&K.

Accession	Location	District	Altitude range
TG-1	Maingal	Chamba	2,500-3,500
TG-2	Bharmour	Chamba	
TG-3	Drobi jangal	Chamba	
TG-4	Baror	Chamba	
TG-5	Hirni	Manali	2,050- 3,050
TG-6	Gojra	Manali	
TG-7	Allain Duhan	Manali	
TG-8	Naggar	Manali	
TG-9	Chandra	Lahaul & Spiti	2,320- 5,000
TG-10	Bhaga	Lahaul & Spiti	
TG-11	Pattan	Lahaul & Spiti	
TG-12	Mayar	Lahaul & Spiti	

The fresh rhizomes of all accessions were washed under running tap water, shade dried, and then grounded to a coarse powder. Plant samples were subjected to Soxhlet extraction using three solvents, a) Hydroalcoholic (30:70; Water: Methanol), b) Ethyl acetate and c) Hexane, based on their polarity according to elliotropic series. Extracts were filtered while hot, concentrated under reduced pressure using rotary evaporator (Heidolf, Germany) followed by lyophilization. Extractive yields were calculated, and extracts were stored at 4°C until used further.

Hydrolysis and quantification of diosgenin by HPLC

Extract hydrolysis was performed for all accessions in accordance to method described by Drapeau et al. (1986). 1500 mg extract was hydrolyzed in 150 ml of refluxing 20% H_2SO_4 in 70% isopropanol for 12 hours. The extracts were then filtered and extracted with hexane (50 ml×3), combined and rinsed thrice with 5% Sodium hydroxide and then rinsed thrice with distilled water. The samples were concentrated by evaporating the solvent at 40°C in a rotary evaporator, and then lyophilized to obtain dried powder. Powder of each extraction was then dissolved in MeOH for HPLC analysis. Diosgenin standard was purchased from Chromadex Inc. (Bangalore, India). A standard stock solution of diosgenin was prepared in HPLC grade methanol and then working solution was made.

Quantification was carried out with reverse phase HPLC (Agilent 1200 series) equipped with HPLC pump, DAD photodiode array detector range from 190-800 nm. Diosgenin was estimated with a Zorbax-Eclipse XBD C-18 3.5 µm (4.6 × 150mm). Solvent system used in gradient mode to run the samples was 0.2% Formic Acid with HPLC grade Water and Methanol in a ratio of 30:70 (v/v). The column was eluted in the gradient mode with a flow rate of 0.8 ml/min. Diosgenin was detected at absorbance of 230 nm wavelength. The cycle time of analysis was 30 min at 25°C with injection volume of 10 µl. At the end of each run, the column was rinsed with pure solvents. The compound was identified on the basis of retention time and comparison of UV spectra with the authentic standard from ChromaDex Inc.

Statistical optimization for maximum diosgenin content

To examine the highest diosgenin content, we took accession with maximum diosgenin content and then various extraction parameters, viz., incubation time (minutes), temperature (°C), and solid-liquid ratio (g/ml) and recorded their cumulative effect by employing RSM using a statistical software package Design Expert 10.0.0.3, Stat-Ease, Inc. A 23 full factorial central composite design (CCD) with 8 trials for factorial design, 6 trials for axial point, and 6 replicate trials at the central point, leading to a set of 20 experiments was designed. The response value from each experiment of CCD was the average of triplicates

Hydrolysis and quantification for diosgenin by high-performance liquid chromatography (HPLC)

Extract hydrolysis of highest diosgenin content accession was performed as described by Drapeau et al. (1986) with modification. Extract volume (solid-liquid ratio), time (in minutes), and temperature (°C) were taken into consideration. Briefly, solid-liquid ratio ranging from 1:100 to 1:200 of extract was hydrolyzed in 150 ml of refluxing with 20% H_2SO_4 in 70% isopropanol for 0-16 hrs at temperature ranging from 70-100°C. The extract was then filtered and extracted with hexane (50 ml × 3). The three hexane extracts were combined and rinsed three times with 5% alkali and then rinsed thrice with distilled water. The extract was then passed through a column of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at 40°C in a rotary evaporator. Rhizome samples were then fractionated with hexane in separating and funnel followed by freezing them in −80°C. Samples were lyophilized and then dissolved in MeOH for HPLC analysis. Diosgenin as standard was purchased from Chromadex, Bengaluru. A standard stock solution of diosgenin was prepared in HPLC grade methanol and then working solution was made. Chromatographic analysis was carried out with reverse-phase HPLC (Agilent 1200 series) equipped with HPLC pump, DAD photodiode array detector range from 190 to 800 nm. Diosgenin was estimated with a Zorbax-Eclipse XBD C-18 3.5 µm (4.6 mm × 150 mm). Solvent system used in a gradient mode to run the samples, i.e. 0.2% formic acid with HPLC grade water and methanol in a ratio of 30:70 (v/v). The column was eluted in the gradient mode with a flow rate of 0.8 ml/min. The diosgenin was detected at absorbance of 230 nm wavelength. The cycle time of analysis was 30 minutes at 25°C with injection volume of 10 µl. At the end of each run, the column was rinsed with pure solvents. The compounds were identified by retention time and comparison of ultraviolet spectra with the authentic standard from ChromaDex, Inc.

Isolation of genomic DNA and total RNA

Genomic DNA was isolated from leaves of *T. govanianum* accessions with highest and lowest content of diosgenin, following the protocol of Murray and Thompson (1980). Total RNA was isolated from rhizomes by using RaFlex Total RNA isolation kit (GeNei™) by following manufacturer's instructions. The quality of RNA was checked in 1% (w/v) ethidium bromide-

stained agarose gel and through absorbance spectrum at wavelengths 260 nm and 280 nm. These were further kept at -80°C until used for cDNA synthesis.

Cloning and sequencing of diosgenin pathway genes in *T. govanianum*

Five enzymes HMG Co-A reductase (*HMGR*), squalene synthase (*SQS*), farnesyl pyrophosphate synthase (*FPPS*), 26-O-beta-glucosidase (*BETA*) and cycloartenol synthase (*CAS*) are well recognized as key enzymes involved in diosgenin biosynthesis pathway (Vaidya et al. 2013; Diarra et al. 2013). Nucleotide and protein sequences for all 5 key genes of diosgenin biosynthesis pathway were retrieved from different plant species in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and multiple sequence alignments (MSA) were done to identify conserved sequence regions. Though the extent of sequence similarity was low in coding regions of genes, short patches of conserved sequences were identified. Primer pairs were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) from conserved regions of gene sequences (Table 1) and tested on genomic DNA and cDNA of *T. govanianum*.

For amplification, the PCR was performed on 30 ng of genomic DNA and cDNA separately with varying amounts of primer pairs, Mg^{2+}, dNTPs and Taq DNA polymerase. Amplification programs included 94°C for 3 min, 30 cycles of 94°C for 30 s, annealing temperature (49-54 °C) for 45 s, 72°C for 2 min and a final extension of 7 min at 72°C. 10 µl of each PCR product was mixed with 2 µl of 6 X gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol) and electrophoresed in a 1.2% agarose gel prepared in 0.5 X Tris acetate-EDTA (TAE) buffer. The gels were analyzed using gel documentation system Alpha Imager EP (Alpha Innotech Corp., USA). PCR products were cloned in pGEMT vector (Promega) and sequenced. Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) was used to calculate sequence similarities. Primer pairs for expression analysis were designed from confirmed gene sequences (Table 2).

Expression analysis of diosgenin biosynthesis pathway genes through quantitative real-time PCR (qRT-PCR)

cDNA was prepared from 5 µg of RNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (GeNei™) with oligo-dT primer. Equal sample quantities were verified by measuring the amount of RNA with a spectrophotometer. The

cDNAs were then separated by electrophoresis, stained with ethidium bromide to further verify equal concentrations. The qPCR was performed using gene specific primers in triplicate on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with the iScript one step RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 55–59 °C, followed by one elongation step for 20 s at 72°C. 26S rRNA and GAPDH were used as internal controls for calculating transcript abundance. The significant differences between treatments were statistically evaluated by standard deviation in the form of error bars as mean ± SD for data recorded in triplicates (repeated thrice).

Anti- cancerous effect of *T. govanianum* extract

MDCK, MCF-7 and MDA-MB-231 were obtained from the NCCS, Pune. Cells were grown in T25 culture flasks containing DMEM (Dulbecco's modified Eagle medium) for MDCK and MCF-7 likewise L-15 (Leibovitz) for MDA-MB-231 supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). MDCK and MCF-7 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching confluency, the cells were trypsinized and passaged.

Cytotoxicity studies

The cytotoxicity studies were performed by MTT assay, according to well established method with slight modification at laboratory conditions (Baydoun et al., 2013; Mosmann, 1983) on three cell lines (MDCK, MCF-7 and MDA-MB-231). The cells were seeded at a density of 1×104 cells/well in 96 well plate and incubated at 37 °C till confluence reached 70%-80%. The cells were then treated with different concentrations of crude extract and 8hr hydrolyzed extract (1ng/ml, 500ng/ml, 1µg/ml, 500 µg/ml, 1mg/ml) were incubated for 24 h. After stipulated time period of treatment MTT (40µl) was added to each well and further incubated at 37°C for 4 hours. Metabolically active cells were able to reduce MTT with the help of enzyme succinate dehydrogenase and form purple colored insoluble formazan. DMSO (100µl) was added to each well to solubilize the formazan crystal and kept at room temperature for 15-20 minutes. Absorbance (A) was taken at 570 nm as test wavelength and 630nm as reference wavelength to

evaluate cell cytotoxicity by using microplate reader (Bio-Rad). Triplicate wells were assayed for each condition and standard deviations were determined.

A decrease in absorbance in this assay measures the extent of decrease in the number of viable cells on exposure to the test substances. The cell cytotoxicity was calculated by using the following formula:

% cell cytotoxicity = $[\frac{\text{Abs test}}{\text{Abs control}} \times 100]$

Acridine orange (AO) and ethidium bromide (ETBR) live dead staining

Further, the best selected concentration was analyzed for its cell morphological changes in MDCK, MCF-7 and MDA-MB-231 cell line by dual AO and ETBR staining. Briefly, cells were seeded at a density of 2 ×105 cells/well in 6 well plate comprising 1ml of growth medium. Till the confluence reached 70-80% cells were incubated at 37°C in CO_2 incubator. Cells were then treated with the best selected concentration and incubated for 24h. After desired time period the cells were harvested, washed with ice-cold PBS and fixed with 4% of para-formaldehyde for 30 min. Afterwards, the supernatant was discarded and cells were treated with 1% triton X for 15min followed by washing of cells with PBS. Cells were stained with dye mixture; comprising 5µL of acridine orange (1 mg/mL) and 5µL of ETBR (1 mg/mL) for 5 min and then washed thrice with PBS. The stained cell with morphological changes were observed by fluorescence microscope at 200 X (Nikon Eclipse-80i, Japan).

Anti-fertility studies of extract of *T. govanianum*

Anti-implantation activity

Female Wistar rat were housed with male rat in the ratio of 2:1 in the evening of proestrous stage and examined next day morning for the evidence of copulation. The rats exhibiting the copulation plug or thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day 1 of pregnancy, and those rats were divided into three groups. Group I served as control and received only vehicle (CMC, 0.3% w/w in saline). Groups II and III rats received the extract orally at the dose level of 125 and 250 mg/kg from day 1 to day 10 of pregnancy. On 11th day, laparotomy was performed under anesthesia using sterile conditions and uteri were examined to determine the number of implantation sites. Weight gained by each rat of all groups was also recorded (Khanna and Chaudhary, 1968; Mohamed et al., 2012).

Results and Discussion

Optimization of diosgenin extraction in different accessions of *T. govanianum*

Hydrolysis and quantification of diosgenin by HPLC

Extractive yield was found to be in the range of 33.26-43.56%, 22.41-43.82% and 26.37-47.21% in hydroalcholic, hexane and diethyl ether, respectively (Fig. 4). HPLC observations revealed that diosgenin content was highest in hydroalcholic extract of TG-1 sample (2.4%), which was collected from Chamba region of Himachal Pradesh. Comparatively hydroalcholic extract showed higher levels of diosgenin content as compared to hexane and diethyl ether extracts. Diosgenin content was found to be in the order, hydroalcholic extract > hexane > diethyl ether (Fig. 5). This can be explained on the basis of the polarity of solvents used in extraction. The level of diosgenin was observed to be higher in hexane extract as compared to diethyl ether (having higher polarity). Hydroalcholic extraction is a well established method to extract compounds of all polarity from the plant samples (approx. 95% extraction) which might have resulted in highest amount of diosgenin in this extract (Fig. 6).

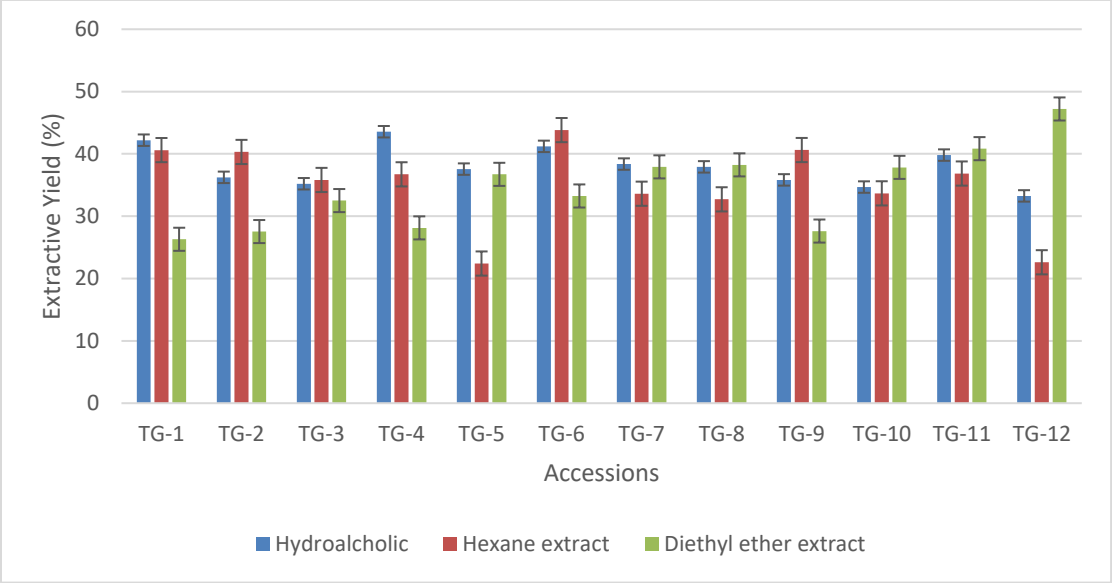


Fig 4. Percent Soxhlet extractive yield in TG-1 - TG-12 accessions of *T. govanianum*

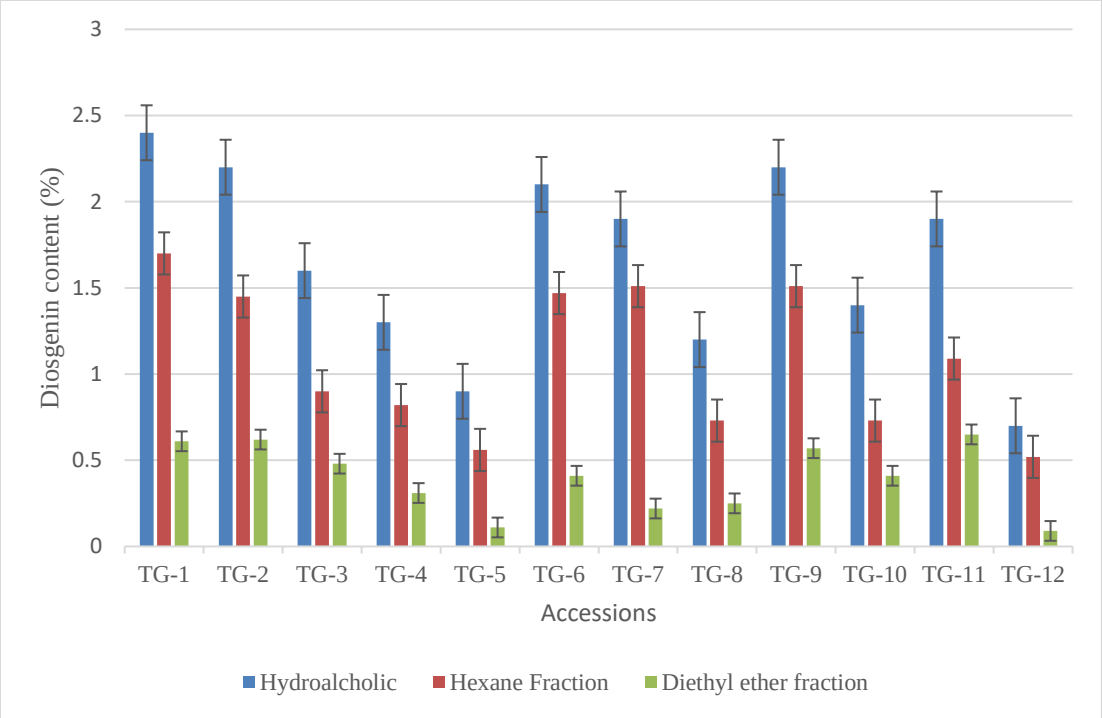
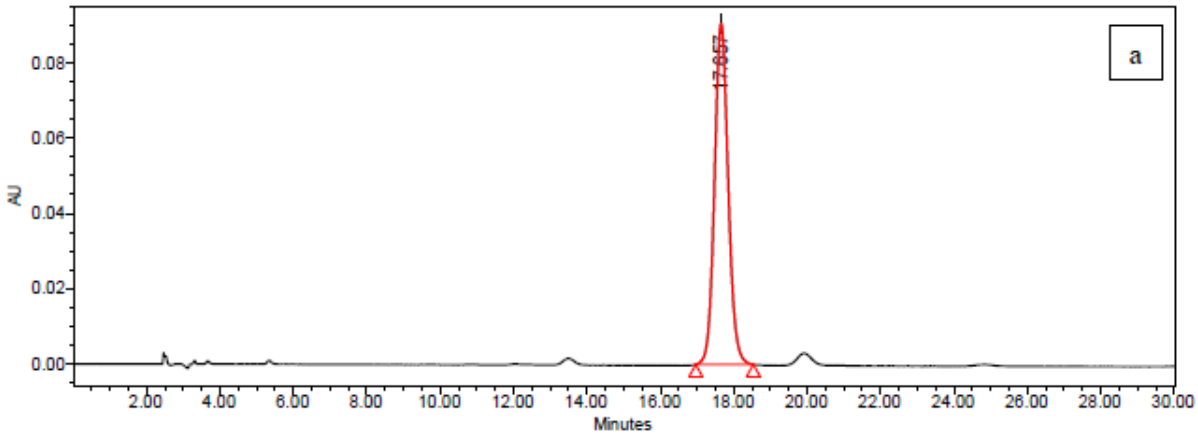


Fig 5. Percent diosgenin content in the hydrolyzed hydroalcholic, hexane and diethyl ether extract of *T. govanianum* accessions, TG-1 –TG-12



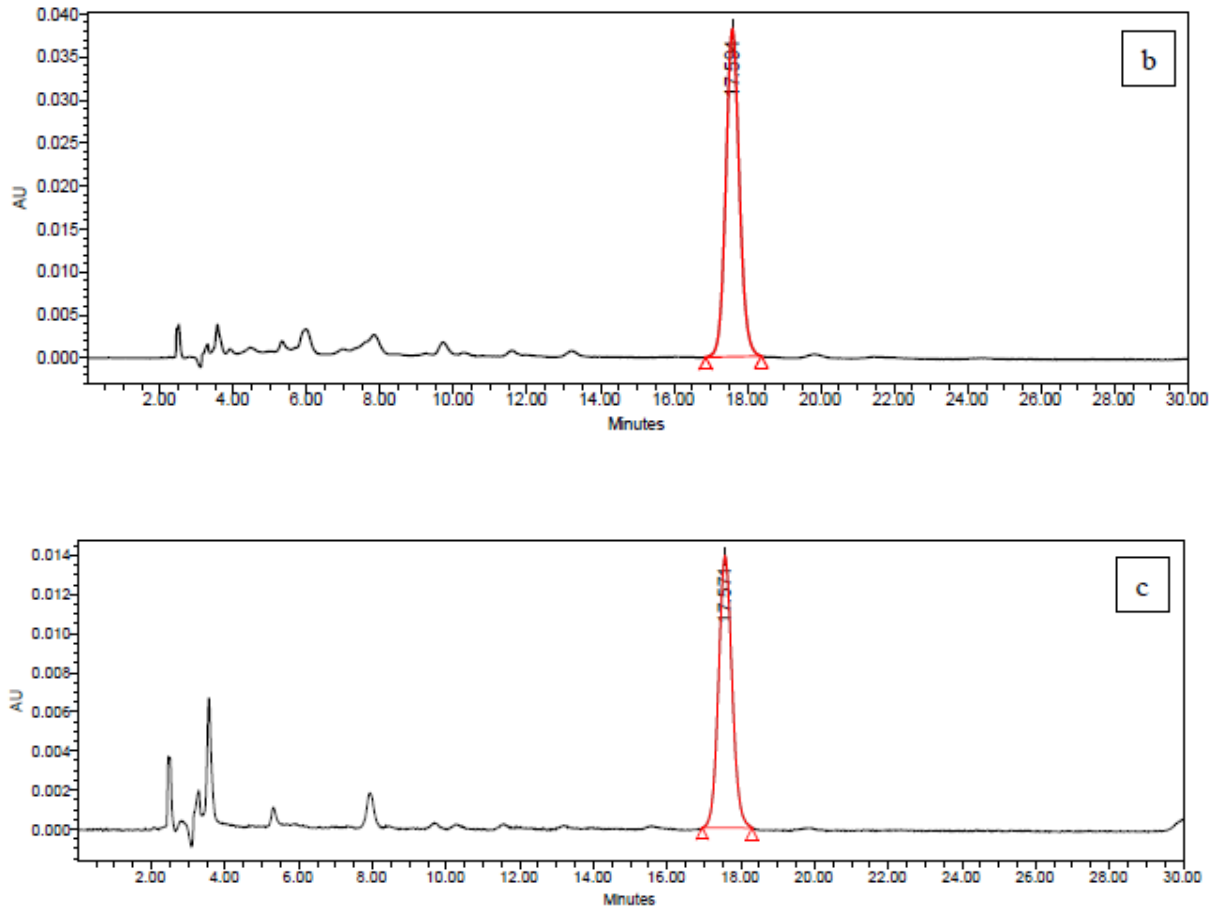


Fig. 6 HPLC chromatograms of diosgenin standard (a) rhizome samples of *T. govanianum* from Chamba (b) and Lahaul & Spiti (c)

Statistical optimization for maximum diosgenin content by response surface methodology

Chamba samples (Maingal area TG-1) having highest diosgenin content (2.4% diosgenin) was taken for the optimization using RSM. The three factors and lower, middle and upper design points for RSM along with the experimental results of diosgenin content (%) are shown in Table no. 4. A polynomial quadratic equation for the total diosgenin content (%) was predicted from multiple regression coefficient determined by employing least squares technique. The polynomial equation was Diosgenin content (%) = 4.97 +2.27A-0.22B+0.70C-0.14AB+0.14AC+0.061BC-1.89A2-10.94B2-0.25C2 where A is incubation time (hr), B is temperature (^{0}C) and C is solid-liquid ratio (gm/mL). The model equation was statistically significant as determined by Analysis of Variance

(ANOVA). The level of variability in the model was explained by coefficient of determination (R^2) which was found to be 0.87 in our model. The cumulative effect of the selected variables i.e incubation time, temperature and solid-liquid ratio are represented as contour plots (2D) as shown in (Fig. 8) which revealed that total diosgenin content was minimum at low and high levels of incubation time and temperature. The contour plot showed that the maximum yield of diosgenin was obtained when hydrolysis was carried out at 85^{o}C for 12 hrs. In contrast to this (Fig. 8b) showed maximum diosgenin content at high levels of solid-liquid ratio while minimum at low and high levels of incubation time. From (Fig. 8c) it was concluded that when temperature was at a certain value the diosgenin content increased with the increase of solid-liquid ratio. Further, the experimental values determined by the experiments were in close agreement with the statistically predicted values which validated the experimental model. Thus, using RSM optimized conditions (incubation time: 12hrs, temperature: 85^{o}C and solid-liquid ratio: 1:234 g/mL), the maximum diosgenin content was 5.99% which was highest reported till date.

Table 2: Factors and levels in the response surface central composite design arrangement along with the experimental and predicted results for Diosgenin content (%)

No.	X1	X2	X3	Experimental	Predicted
1	0	100	200	0	0.16
2	21.45	85	150	2.2	3.45
3	0	70	100	0	0
4	8	85	150	5.1	4.96
5	8	59.77	150	2.1	2.67
6	8	85	150	5.1	4.96
7	8	85	65.91	1	3.09
8	8	85	150	5.1	4.96
9	16	70	200	5.3	5.29
10	0	85	150	0	0.80
11	8	110.22	150	1	1.96
12	8	85	234.09	5.99	5.43
13	16	70	100	4.99	3.74
14	16	100	200	5	4.71
15	8	85	150	5.1	4.96
16	8	85	150	5.1	4.96
17	0	70	200	0	0.19

18	0	100	100	0	0
19	16	100	100	4.2	2.91
20	8	85	150	5.1	4.96

X1= Incubation Time (hr); X2= Temperature (degree C) X3= Solid-Liquid Ratio (gm/mL)

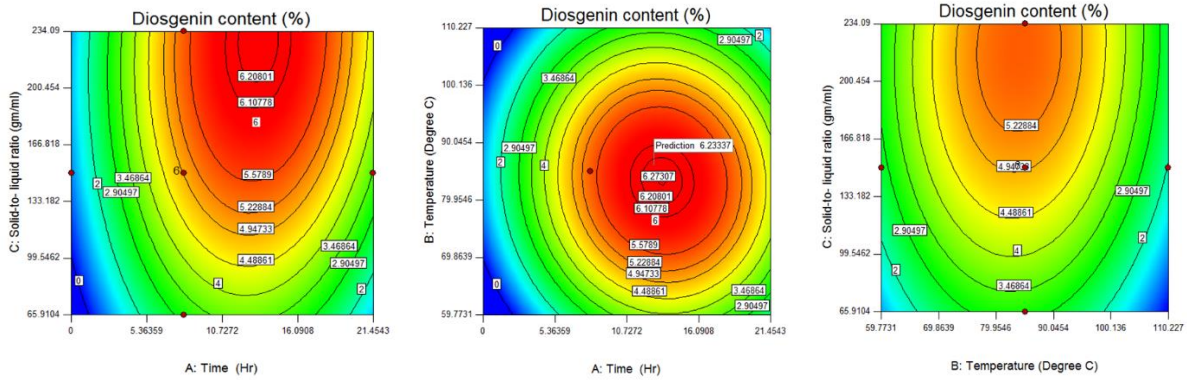


Fig. 7. Effect of (a) Time and Temperature (b) Time and Solid-Liquid ratio and (c) Temperature and Solid-Liquid ration on the yield of Diosgenin (%)

Discerning the expression status of steroidal pathway genes vis-a-vis diosgenin content in different accessions of *T. govanianum*

Cloning steroid pathway genes in T. govanianum

Partial gene sequences of 5 genes encoding key enzymes involved in diosgenin biosynthesis pathway were cloned from *T. govanianum* through comparative genomics using primer pairs designed from conserved gene regions (Table 2). The amplicons for all gene fragments, ranging from 300-600 bp, were sequenced and annotated for biological function. The sequences were utilized for designing primers for gene expression analysis (Table 3).

Table 3. Primer sequences used to clone five genes of diosgenin biosynthetic pathway in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature
HMGR	CTTCCGATGTTTTACCTTTG	GCAGGCTTCTCTATGATGAC	52

SQS	TTGAACGACATGGTCACTAA	CATTGTAGATGTTGGTGCAG	54
FPPS	AATGTCTGGTGAAGATTTGG	AAGAGATCAGCTGCTCGTAG	52
BETA	AGTCTGAGCTGATCAAAGGA	TGAACCACTTGGTAGAATCC	50
CAS	GGTTTACATATCGAGGGTCA	GAGCAAGGTTCCAGTCTATG	49

Table 4. Gene specific primers used for qRT-PCR analysis in *T. govanianum*

Gene	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Annealing Temperature
26S rRNA	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56
HMGR	AACGTCCTGGATTTCCTTCA	TCTTCACCACCTCCTCCTTG	55
SQS	ACTCAGTCAAGGCAGTGCAA	GCAGTAAGACCACGCCTCAT	59
FPPS	GGTTTAGGGTGCCTCAGGTT	GCCCTGAAGCTGTCTTGAAC	55
BETA	GAATTCGGGGACAGAGTGAA	GAGGTTGTGGGTGACTTCGT	55
CAS	AGTGCACCTCGGCATCTATC	TACAGCCCAAGAGCCATACC	55

Expression analysis of diosgenin biosynthetic pathway genes

The qRT-PCR analysis revealed that all 5 genes viz. *HMGR, FPPS, SQS, BETA* and *CAS* involved in diosgenin biosynthesis had relatively higher expression in the rhizomes of Chamba (2.4%) (upto 3.2 folds) ($p < 0.05$) as compared to Lahaul & Spiti (0.7%) (Fig. 7). These results could be positively correlated to the findings of Mehrafarin et al. (2010) and Vaidya et al. (2013) which have shown the importance of these genes for diosgenin production in *Trigonella foenum graecum*. Since *HMGR* catalyzes the first committed step of isoprenoid biosynthesis in mevalonate pathway (Wang et al. 2007), it showed highest expression of 3.2 fold ($p < 0.05$) compared to all other genes in *T. govanianum*. The enzyme coding for *CAS* was up regulated by 2.5 fold ($p < 0.05$). It has been known that *CAS* plays a vital role in plant cell viability and regulation of triterpenoid biosynthesis. Further, enzymes coding for *SQS*, *FPP* and *BETA* were up regulated by 1.3 fold ($p < 0.05$), 2.1 fold ($p < 0.05$) and 1.7 fold ($p < 0.05$), respectively. They are also known to be involved in steroid

biosynthesis (Vaidya et al. 2013). *SQS* is a bifunctional enzyme that catalyzes the condensation of two molecules of farnesyl pyrophosphate to squalene, a key precursor for sterol and triterpene biosynthesis in plant cells (Huang et al. 2007; Lee and Poulter 2008).

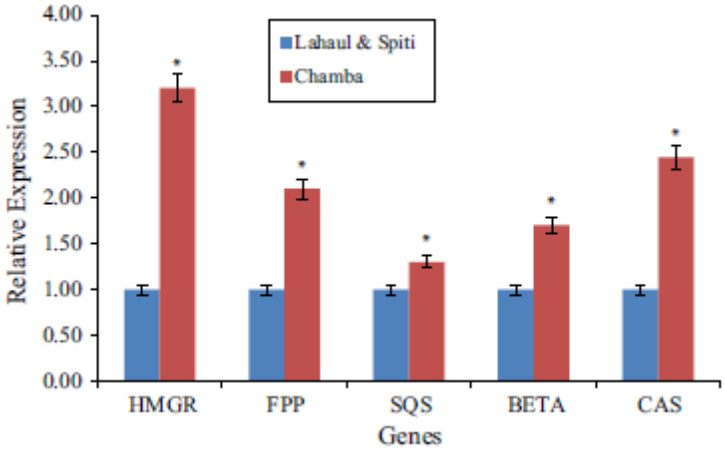


Fig. 8 Relative expression of key genes involved in diosgenin biosynthesis pathway in rhizomes of *T. govanianum* from Chamba (2.4%) and Lahaul & Spiti (0.7%). Error bars represent mean ± SD for data recorded in triplicates (repeated thrice). Significance was evaluated for each gene between both samples (*****$p < 0.05$).

Anti- cancerous effect of T. govanianum extract on cell viability

Crude rhizome extract of *T.govanianum* showed less toxicity on MDCK cells (Fig. 4a) with IC-50 value of 2.1 µg/ml and cell viability of MCF-7 and MDA-MB-231 cells were having IC50 value 2.03 and 1.8 µg/ml respectively. Likewise, hydrolyzed extract of *T. govanianum* were less toxic in MDCK cells (Fig 9a) with IC-50 value of 1.7 µg/ml but significantly reduces proliferation of MCF-7 (Fig. 9b) and MDA-MB-231 cells (Fig. 9c) with IC-50 value 1.11 and 0.495 µg/ml respectively. This suggests that the extract is less toxic against normal cells but showed a significant anti-proliferative activity on cancer cells (MCF-7 and MDA-MB-231). On the contrary, hydrolyzed extract is also having a less toxicity towards normal cells, whereas considerably reduces cancer cell viability. Cytotoxity of diosgenin has been previously established on many cell lines like V79 fibroblast and K562 cells (Liu et al. 2005; Melo et al. 2004). Our results were consistent to previously reported findings which did not show any significant toxicity on MDCK but comparatively effective on MCF-7 and MDA-MB-231. This may be due to the presence of

diosgenin and other steroidal, saponins compounds in *T. govanianum* as they have been reported previously for their anticancer activity (Ur Rahman et al. 2015).

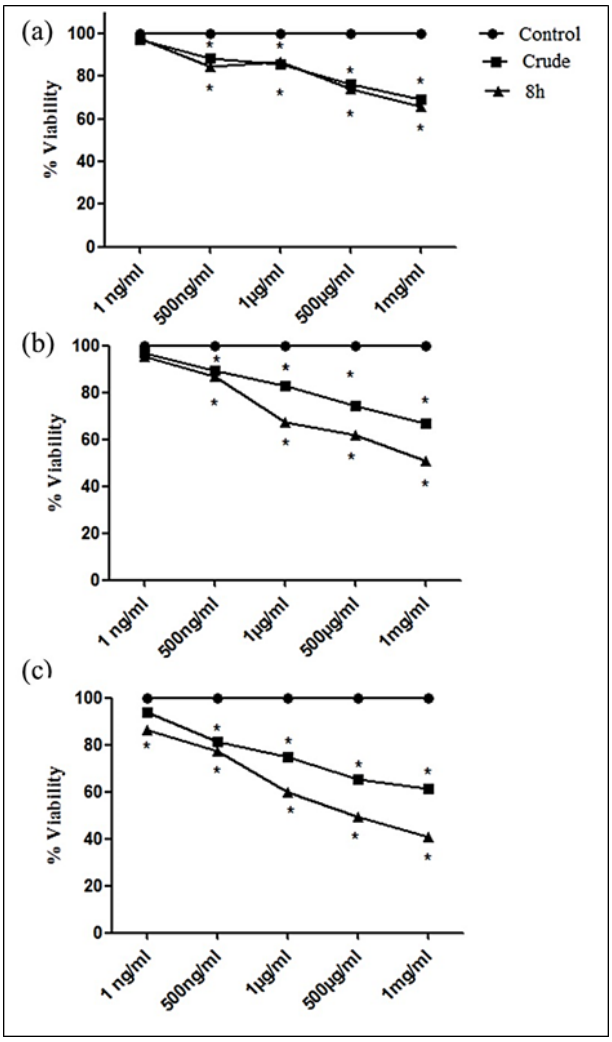


Fig. 9. Inhibition of proliferation in MDCK (a), MCF-7 (b) and MDA-MB-231(c) cells after treatment. Results are presented as percentage of control (Untreated cells) and values are expressed as means ± SD of six experiments (* p-value relative to control group, $p<0.05$).

Cell and nuclear morphology

Morphological analysis for MDCK, MCF-7 and MDA-MB-231 cells were performed and photographs were taken under fluorescence microscope at 200 X (Nikon Eclipse-80i, Japan). The cells were treated with respective IC-50 value of crude extract and 8hr hydrolyzed extract concentration for 24 hrs and further compared with untreated cells using acridine orange/ ethidium bromide (AO/ETBR) dual staining. AO/ ETBR is generally used to examine nuclear morphological changes in apoptotic cells. Acridine orange dye is well reported, as it binds to both live and dead cells, whereas ethidium bromide stain those cells which loses their membrane integrity. Cells stained with green fluorescence represent the viable cells, whereas the yellow

staining signifies early apoptotic cells, and orange cell denotes late apoptotic cells. Likewise, in untreated control cells, uniform green fluorescence was observed which signifies the cell with normal nuclear morphology. Were as in treated cells yellow and orange stains were observed (Fig. 10, 11, 12).

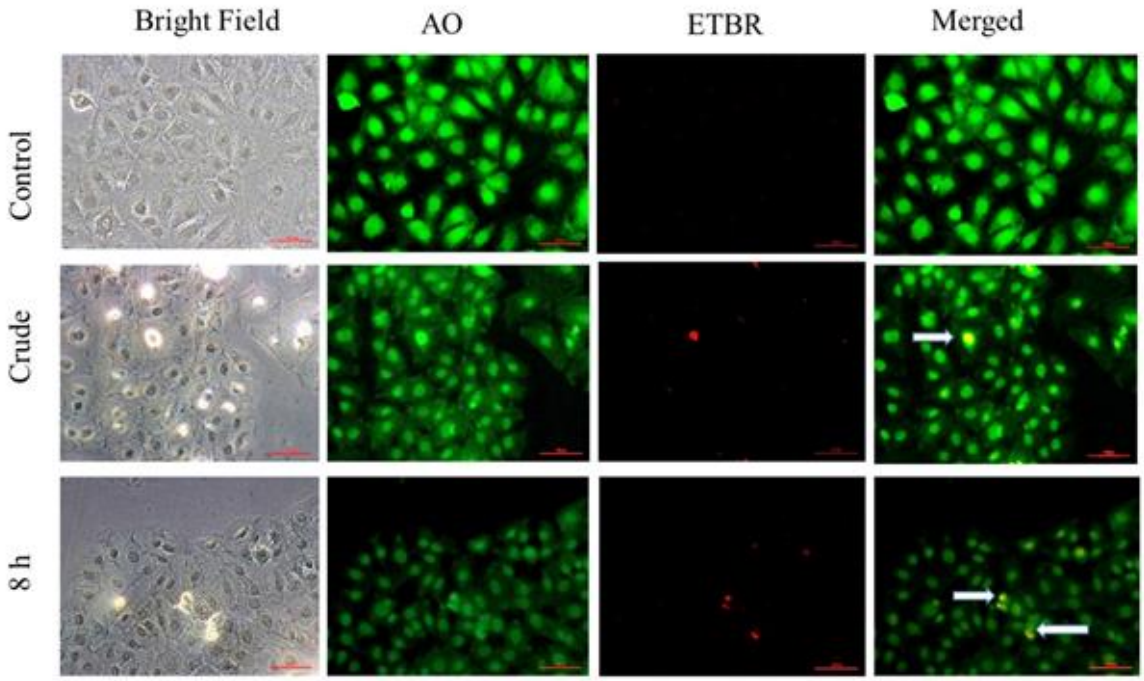


Fig. 10. Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDCK cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X).

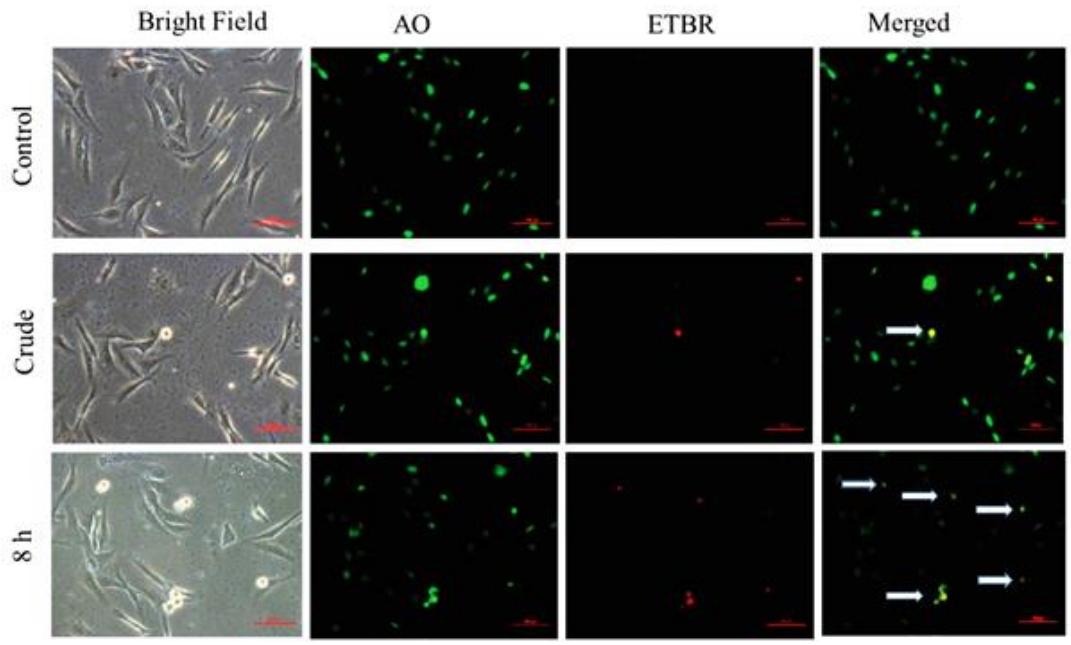


Fig. 11. Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MDA-MB-231 cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X).

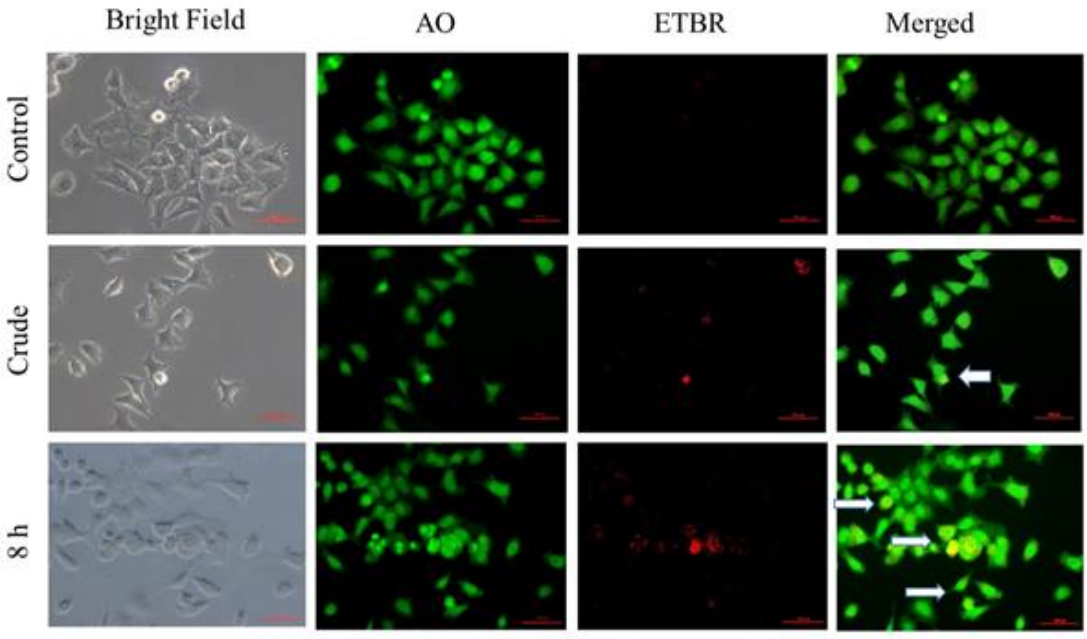


Fig. 12. Effect of *T. govanianum* crude extract and hydrolyzed extract on cell and nuclear morphology in MCF-7 cells through AO/ETBR staining, viewed under a fluorescence microscope (200 X).

Anti- fertility studies of *T. govanianum* rhizome extract

Anti-implantation activity

The *T. govanianum* showed dose dependent anti-implantation effect and the results are shown in (Table 5). 100% implantation inhibition was observed at 125 and 250 mg/kg extract treatment. This strong anti-implantation effect may be due to the high estrogenic like activity of the plant extract. The estrogen levels were quantified in serum and were observed to be 106.72 and 216 pg/ml in the animals treated with 125 and 250 mg/kg extract respectively, compared to normal animals having estrogen level of 100.84 pg/ml (Table 5)

Table 5: Effect of plant extract on implantation in female rats.

Treatment	Dose (mg/kg)	Anti-implantation effect			
		% change in Body weight (mean)	No. of implantation sites (mean)	% Inhibition of implantation sites on day 11	Estrogen (pg/ml)
Control	-	5.48%	11	0%	100.84
Plant Extract	125	1.64%	0	100%	106.72
	250	8.09%	0	100%	216

Hispathological examination of ovaries and uterus

We performed histopathological examination of uterus and ovaries of extract treated and control animals. Results of the histopathology are depicted in figure 13 and 14. Histopathological examination revealed healthy morphology of uterus and ovaries in control animals. However, treating animals with extract resulted in thickening of endometrium in uterus in dose dependent manner, which might have interfered with the implantation of fetus on to the endometrium wall. Morphology of ovaries were normal in extract treated animals and showed no signs of any damage.

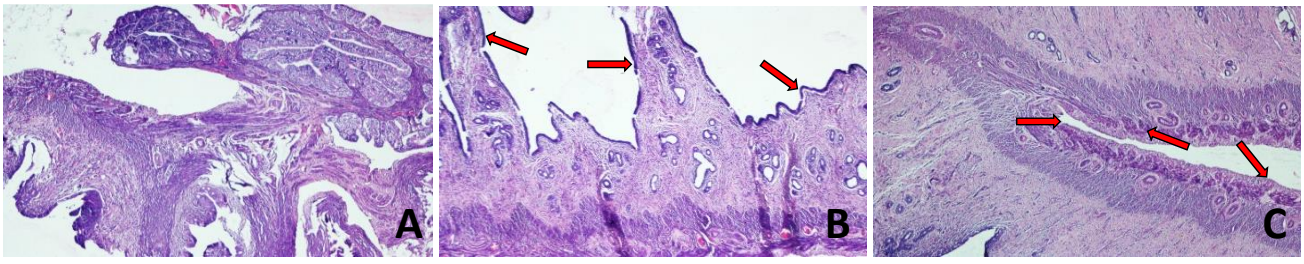


Fig. 13. Histological sections of uterus of the control (A), extract 125 mg/kg (B) and extract 250 mg/kg (C).

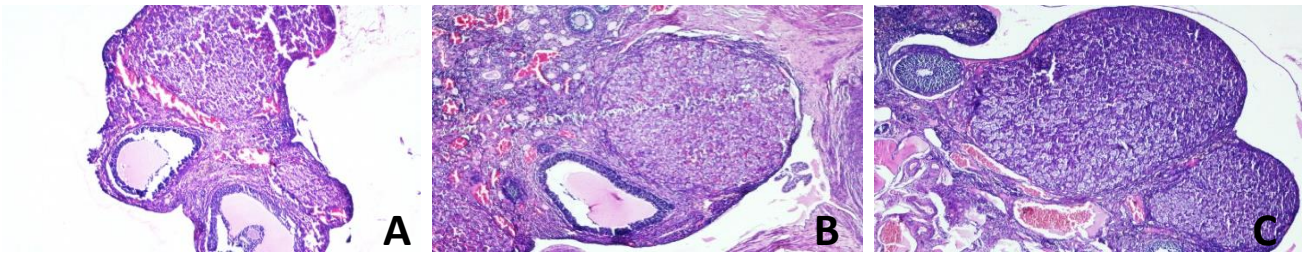


Fig. 14. Histological sections of ovary of the control (A), extract 125 mg/kg (B) and extract 250 mg/kg (C).

Conclusion and future prospects

Diosgenin biosynthesis was found to be regulated by the key enzymes identified and the expression analysis of multiple genes revealed their primary role which would be helpful in successful exploitation of plant metabolism for enhancement of diosgenin biosynthesis. Optimization of extraction process for diosgenin might help the pharmaceutical sector in a great way for large-scale purification. Further, trillium extract was found to be an efficient anti-cancerous effect against MCF-7 and MDA-MB-231 cells. Anti- implantation studies of the extract of *Trillium govanianum* showed promising leads as an effective herbal contraceptive with zero implantation.

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LIST OF PUBLICATION

Journal Publications:

1. **Shivam Sharma**, Nikhil Malhotra and Hemant Sood (2016). Expression Analysis of Steroid Pathway Genes Revealed Positive Correlation with Diosgenin Biosynthesis in *Trillium govanianum*. ***Acta Physiologiae Plantarum*** **[IF: 1.5]. [SCOPUS]**
2. **Shivam Sharma**, Arun Sharma, Vineet Mehta, Rajinder S. Chauhan, Udayabanu M, Hemant Sood (2016). Efficient Hydroalcoholic Extraction for Highest Diosgenin Content from *Trillium Govanianum* (Nag chhatri) and its in Vitro Anticancerous Activity. ***Asian Journal of Pharmaceutical and Clinical Research***, 9 (4), 386-392. **[IF : 0.5]**. **[SCOPUS]**

Conference:

Shivam Sharma, Varun Kumar and Hemant Sood (2016) "Optimization of extraction protocol for diosgenin by using response surface methodology (RSM) from *Trillium govanianum*" at International conference on "Biodiversity: Current Scenario and Future Strategies" sponsored by "State Council for Science, Technology and Environment Himachal Pradesh" held at St. Bede's College, Shimla, October 6-8

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